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A VALIDATED RP-HPLC METHOD FOR THE SIMULTANEOUS DETERMINATION OF LEVODOPA, CARBIDOPA AND ENTACAPONE IN TABLET DOSAGE FORMS

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ABSTRACT

A Stability indicating RP-HPLC method has been developed for the simultaneous estimation of Levodopa, Carbidopa and Entacapone in tablet dosage forms using an RP- Hypersil BDS column (150 x 4.6 mm; 5µ). A mobile phase consisting of Potassium Dihydrogen orthophosphate and Acetonitrile in the ratio of 95:5.0 was pumped through the column at a flow rate of 1.0 mL/min. The separations of drugs were monitored at 280 nm. Linearities of quantification were observed in the concentration range of 75–225 µg/ml for Levodopa, 18.75-56.25 µg/ml for Carbidopa and 100 -300 µg/ml for Entacapone. The method was duly validated. A%RSD of less than 2% indicates the high degree of accuracy and precision of the method. Due to its high precision and accuracy, the proposed Stability indicating RP-HPLC method can be applied for determining Levodopa, Carbidopa and Entacapone simultaneously in bulk and Combined Tablet dosage forms.

KEYWORDS: Levodopa, Carbidopa and Entacapone; RP-HPLC Method; Forced Degradation studies; Tablet dosage forms.

INTRODUCTION

Stalevo® (carbidopa, levodopa and entacapone) is a combination of carbidopa, levodopa and entacapone for the treatment of Parkinson's disease.

1. **Levodopa** is an aromatic amino acid, is a white, crystalline compound, slightly soluble in water, with a molecular weight of 197.2. It is designated chemically as (-)-L-α-amino-β-(3,4 dihydroxybenzene) propanoic acid. Its empirical formula is $C_9H_{11}NO_4$, and its structural formula is shown in **fig.1.**

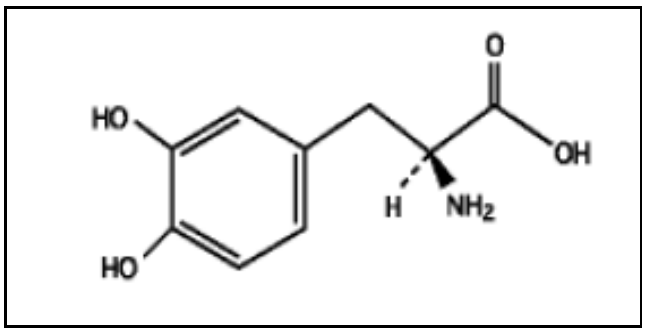


Figure 1. Chemical structure of Levodopa.

2. **Carbidopa** is an inhibitor of aromatic amino acid decarboxylation, is a white, crystalline compound, slightly soluble in water, with a molecular weight of 244.3. It is designated chemically as (-)-L-(α-hydrazino-(α-methyl-β-(3,4-dihydroxybenzene) propanoic acid monohydrate. Its empirical formula is $C_{10}H_{14}N_2O_4 \bullet H_2O$ and its structural formula is shown in **figure.2.**

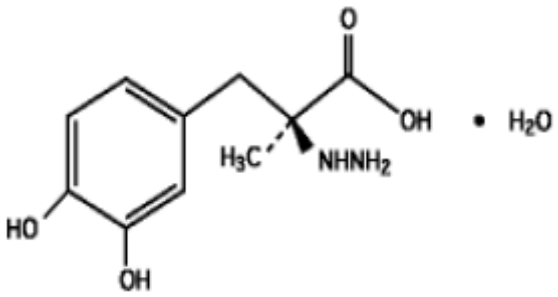


Figure 2.Chemical structure of Carbidopa.

Tablet content is expressed in terms of anhydrous carbidopa, which has a molecular weight of 226.3.Tablet content is expressed in terms of anhydrous carbidopa, which has a molecular weight of 226.3.

3. **Entacapone** is an inhibitor of catechol-O-methyltransferase (COMT), is a nitro-catechol-structured compound with a molecular weight of 305.3. The chemical name of entacapone is (E)-2-cyano-3-(3,4-dihydroxy-5-nitrophenyl)-N,N-diethyl-2-propenamide. Its empirical formula is $C_{14}H_{15}N_3O_5$ and its structural formula is shown in fig.3.

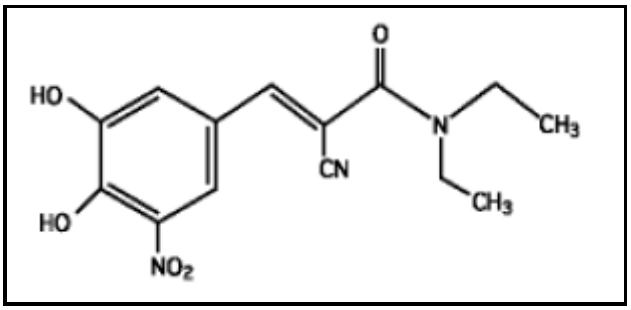


Figure .3.Chemical structure of Entacapone.

In the literature, various analytical methods have been reported for quantitative determination of Levodopa, Carbidopa and Entacapone alone and in combination. However, the reported HPLC[6-9] method for the simultaneous estimation of LDP, CDP and EPN used phosphate buffer which is not LC-MS compatible and the retention times were longer. Hence, the aim of present investigation is to develop a validated Stability indicating RP-HPLC-PDA method for the simultaneous estimation of LDP, CDP, and EPN in bulk and dosage forms which is sensitive, Precise and Accurate.

Reagents and Chemicals

Carbidopa, Levodopa and Entacapone standard drugs were provided as gift samples from Dr. Reddy's laboratories limited, Hyderabad. The branded formulations (tablets) a (Stalevo) containing Levodopa (150mg), Carbidopa (37.5 mg), and Entacapone (200mg) were commercially purchased.) Were procured from the local market. Acetonitrile, Water, Potassium dihyrogen ortho phosphate and orthophosphoric acid used were of HPLC grade and purchased from Merck Specialties Private Limited, Mumbai, India.

Instrumentation

RP-HPLC waters 2695 separation module equipped with 2996 Photodiode Array Detector was employed in this method. The Empower -2 software was used for LC peak integration along with data acquisition and data processing. High performance liquid chromatograph waters 2695 equipped with Quaternary constant flow pump, Auto injector with injection

volume of 10 µl. 2696 Photo diode Array detector and Empower-2 software, Hypersil BDS C_{18} column (150 mm × 4.6 mm I.d.,5 µ size particle) forms the stationary phase,

Chromatographic conditions

Flow rate	**:**	1.0 ml/min
Column	**:**	Hypersil BDS C18, 150 x 4.6 mm, 5µ.
Detector wave length	**:**	280 nm
Column temperature	**:**	30°C
Injection volume	**:**	10µL
Run time	**:**	13 min
Diluent	**:**	Buffer: ACN (1:1)

OBJECTIVE

To develop a simple, accurate and precise RP-HPLC method for simultaneous estimation of Levodopa, Carbidopa and Entacapone, validating the method according to ICH guidelines and testing its applicability for the routine analysis of levodopa, carbidopa and entacapone in combined tablet dosage forms.

EXPERIMENTAL METHODS

OPTIMIZED METHOD

Buffer

Weigh accurately 6.8 gm of potassium Di hydrogen orthophosphate into a 1000ml beaker .add about 900ml of milli-Q water and sonicate and make up to the final volume. Adjust the solution to pH-2.5 with dilute ortho phosperic acid.

Method Development and Optimization

Optimization of UV conditions: The method development work was started by taking UV-spectra in the range 200-400 nm of Levodopa, carbidopa and entacapone (10PPM) standard solution. These samples were scanned in the range 200-400 nm using UV-visible spectrophotometer. By observing the UV spectra of standard solutions, the detection wavelength was selected as 280 nm for trails to develop HPLC method.

Preparation of sample.

Twenty tablets of (Stalevo) were weighed and the average weight of one tablet was determined. The tablets were finely powdered and the powder equivalent to the weight of 5 tablets was weighed (3600 mg) and transferred into a 500 mL volumetric flask. 350 mL of the diluent was added and sonicated for 25 min, and the volume made up with the diluent and

filtered. From the filtered solution, 1.0 mL was pipeted out into a 10 ml volumetric flask and the volume made up with the diluent.

Preparation of Standard Solutions

Accurately Weighed and transferred 75mg of Levodopa ,18.75 mg of Carbidopa and 100mg of Entacapone working Standards into a 50 ml clean dry volumetric flask, add 37ml of diluent, sonicated for 5 minutes and make up to the final volume with diluent(standard stock).

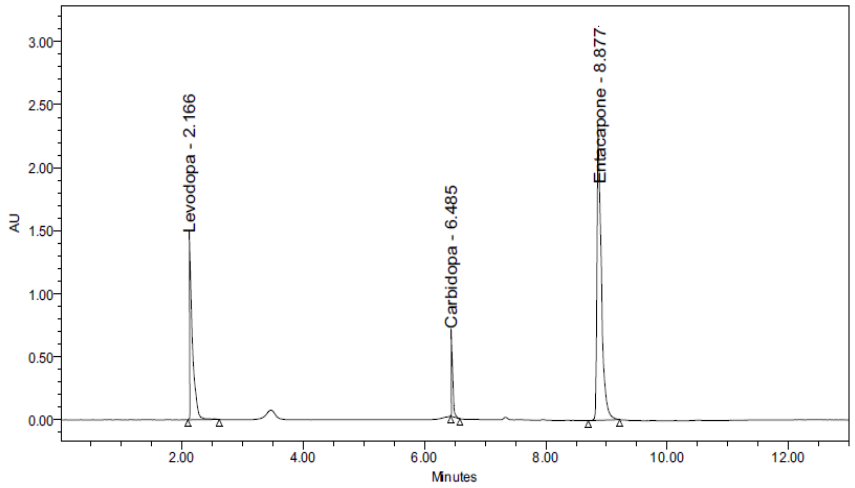
Method development

The proposed method was developed using a mixture of Potassium Dihydrogen orthophosphate and Acetonitrile in the ratio of 95:5.0 (pH 2.5) as the mobile phase in isocratic mode. The flow rate was 1.0 ml/min and the eluate was monitored at 280 nm for detection of the analytes.

The relevant calibration curves were linear in the concentrations ranges of 25-225, 18.75 and 100-300 µg/ml for levodopa, carbidopa and entacapone respectively (Table 1). For analysis of tablet formulation, the tablet powder equivalent to 25 mg was taken, dissolved in 25 ml volumetric flask and made up to 25 ml with Acetonitrile. The solution was sonicated for 15min, centrifuged at 100 rpm for 15 min and filtered through a Whatmann filter paper No.41. From the clear solution, further dilutions were made to get 10 µg/ml of levodopa, carbidopa and entacapone theoretically. For recovery studies, to the pre-analyzed formulation samples, different concentrations of pure drug solutions were added and the amounts of drugs recovered were calculated by using the calibration graphs. Finally the method was validated as per ICH guide lines for precision, accuracy, specificity, linearity, limit of detection and limit of quantification.

Table No.1.Linearty values of Levodopa, Carbidopa, and Entacapone.

S.No	Pipetted from stock (mL)	Volume of flask (mL)	Con of Levodopa Ppm	Con of Carbidopa ppm	Con of Entacapone ppm	% of linearity level
1	0.5	10	75	18.75	100	50
2	0.75	10	112.5	28.125	150	75
3	1	10	75	18.75	100	100
4	1.25	10	187.5	46.875	250	125
5	1.5	10	225	56.25	300	150



	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing
1	Levodopa	2.166	3406809	18.06	4702	1.06
2	Carbidopa	6.485	269210	1.43	95450	1.40
3	Entacapone	8.877	15189623	80.51	117948	1.57

Fig.4.Optimum Chromatogram for mixture of Levodopa, Carbidopa, Entacapone.

Method Validation: The HPLC method was validated in according to ICH guidelines[10-11] for validation of analytical procedures for different validation parameters. The method was validated for its specificity, linearity, accuracy, precision, robustness, ruggedness, LOD and LOQ.

RESULTS AND DISCUSSIONS

In this work, an isocratic, accurate and sensitive HPLC method suitable for the simultaneous determination of Levodopa, Carbidopa and Entacapone in pure form and in pharmaceutical formulations using a 150 mm x 4.6 mm, i.d. Hypersil ODS C_{18} 5µ analytical column has been developed. The mobile phase was chosen after several trials to match the optimum stationary/mobile phase. The present method contains mobile phase phosphate buffer (pH-2.5): Acetonitrile (90:05 v/v) which was found to be the most suitable, as the chromatographic peaks obtained were better defined, well resolved and almost free from tailing. The flow rate is 1 ml/min. The average retention times under the conditions described were minutes for Levodopa, Carbidopa 2.17, 6.48 minutes and 8.863 for Entacapone. The total run time is 13 minutes with which all the system suitability parameters are ideal for the mixture of standard solutions.

System suitability: System suitability test was carried out to verify that the analytical system is working properly and can give accurate and precise results. The overall system suitability

was evaluated for the system suitability of the proposed method. Data from six injections (10µg/mL) were utilized for calculating parameters like theoretical plates, resolution, tailing factor and %RSD of 6 injections.

Specificity: The specificity studies were carried out by varying specific conditions i.e., placebo study. Specificity of the method was established by demonstrating that there was no interference from the excipients. This was demonstrated by preparing the placebo containing all excipients except the drug and also the sample prepared from the same. The samples were injected individually and chromatogram was obtained.

Linearity: Linearity of an analytical procedure is its ability (within a given range) to obtain test results that are directly proportional to the concentration (amount) of analyte in the samples 23. The linearity of the method was determined by preparing serial dilutions of minimum 5 concentration of working stock solutions in the range of 17-225, 18.75-56 µg/ml for Levodopa, Carbidopa and 100-300 µg/ml for Entacapone. The area of each injection was obtained and the peak area was plotted against actual concentration. The regression coefficient 'r^2', y-intercept and slope of the regression were calculated. Results were represented on figure 5, 6 and 7.and Table 2.

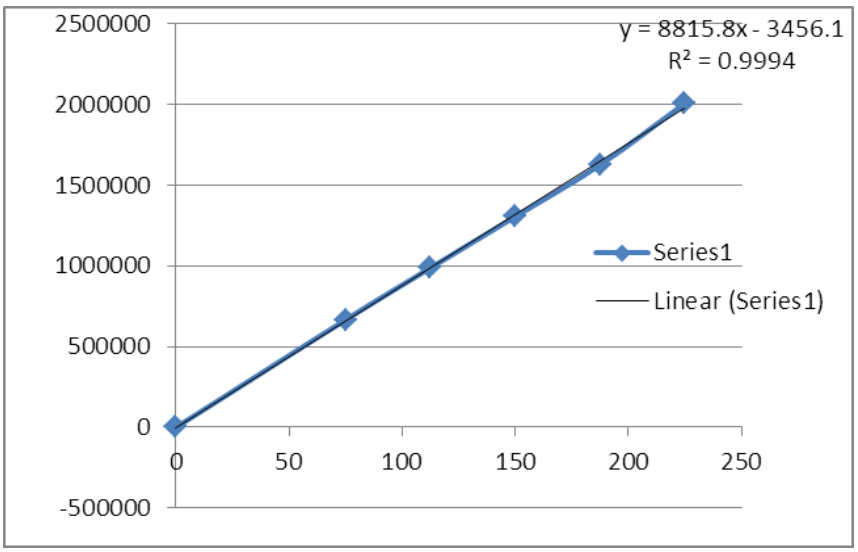


Figure.No.5 Calibration graph of Levodopa

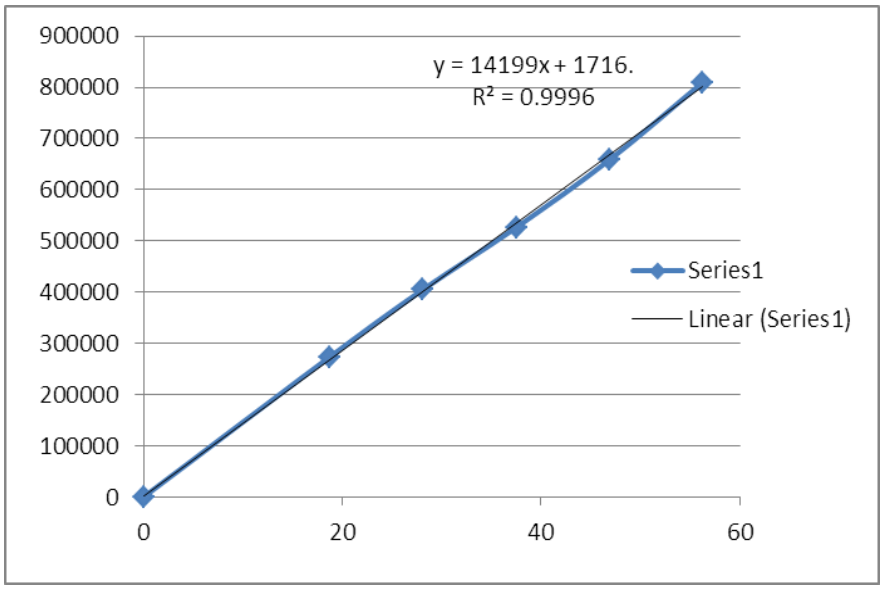


Figure 6.Calibration curve of Carbidopa

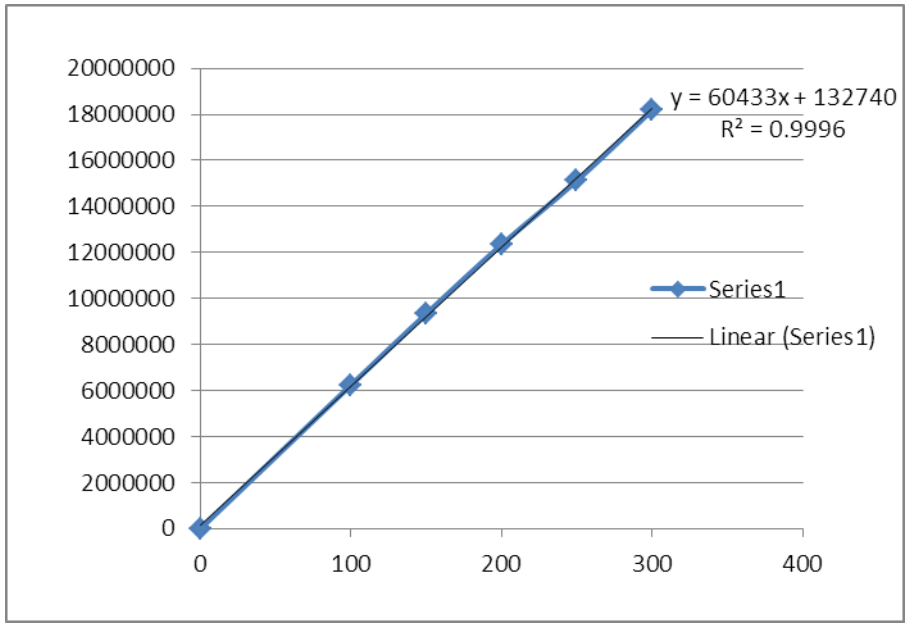


Figure 7.Calibration curve of Entacapone

Table 2. Linearity Results

S.No	**Concentration (µg/mL)**			**Peak Area**		
	LDP	CDP	EPN	LDP	CDP	EPN
	75	18.75	100	665968	273680	6237108
	112.5	28.125	150	987339	405089	9339241
	150	37.5	200	1305575	526295	12343694
	187.5	46.875	250	1625391	658295	15119727
	225	56.25	300	2006809	809210	18189623

Limit of Quantity and Limit of Detection: The LOD is defined as the lowest concentration of an analyte in a sample that can be detected but not quantified. The LOQ is defined as the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy. The LOD and LOQ can be calculated based on the standard deviation of the response and the slope of calibration curve. The Values are Shown by Table 3.

Table 3 LOD and LOQ values

S.No	Levodopa	Carbidopa	Entacapone
LOD (s/n ratio)	3.2	3.4	3.1
LOQ (s/n ratio)	9.2	10.2	10.00

Accuracy: Accuracy was carried out by % recovery studies of LDP and CDP and EPN at three different concentration levels (50%, 100%, 150%). In the proposed method recovery studies were carried out by collecting the sample solution of 20 tablets containing LDP and CDP and EPN and analyzed. Percentage of recovery was calculated from the amount added and amount recovered. The percentage recovery was within the acceptance criteria, this indicates the accuracy of the method. (Acceptance criteria: % recovery between 98 to 102).

Table 2. Recovery Studies of Levodopa, Carbidopa and Entacapone.

Drug Name	Level of % Recovery	% Mean Recovery*	S.D	% R.S.D*
Levodopa	50 100 150	98.66 100.16 99.82	0.43 1.36 1.35	0.44 1.35 1.35
Carbidopa	50 100 150	100.02 99.91 100	1.102 0.170 428	1.102 0.171 1.428
Entacapone	50 100 150	100.04 99.69 99.88	1.042 1.274 1.06	1.042 1.274 1.06

***Avg. of three determinations for 50, 150 &100%,**

R.S.D. is relative standard deviation

Precision: Precision of an analytical procedure as the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample under the prescribed conditions 23. It has done by the following methods.

System precision (Reproducibility): System precision of method was carried out by measuring the peak response of LDP and CDP and EPN for six replicate injections of standard solution. The retention time and area ratio of six determinations were measured and percent coefficient variation (% RSD) calculated. (Acceptance criteria: % RSD not more than 2%). The values were represented Table 4.

Table 4 System precission

No. of injections	Peak Area		
	Levodopa	Carbidopa	Entacapone
1. Injection	1339842	537516	12508101
2. Injection	1324974	535429	12395770
3. Injection	1351580	536151	12810757
4. Injection	1388298	534357	12159907
5. Injection	1361448	541507	12493026
6. Injection	1319983	548971	12591001
Average	1347688	538988.5	12493094

Method precision: It can be done by 2 methods.

Repeatability (Intraday precision): Repeatability was carried out by analyzing six replicate injections of assay concentration of standard and sample solutions over a short time interval (within a day) under same conditions. The percentage relative standard deviation (% RSD) was calculated for the resultant peak areas. (Shown on Table.5).

Table .5.Intraday Precision

No.of injections	Peak Area		
	Levodopa	Carbidopa	Entacapone
1. Injection	98.23	99.04	100.71
2. Injection	99.98	101.71	101.54
3. Injection	100.34	100.4	98.74
4. Injection	101.34	99.8	99.03
5. Injection	100.96	99.11	101.12
6. Injection	101.33	99.77	98.68
Average	100.3633	99.97167	99.97
S.D	1.178383	0.988522	1.295809
%RSD	1.174117	0.988803	1.296197

Intermediate precision (Inter day precision): Intermediate precision was assessed by analyzing the same standard and sample solutions on different days. % RSD of assay results were calculated. To assess the degree of reproducibility of the method was analyzed on different day. The assay procedure was repeated six times and the chromatogram was recorded and the %RSD was calculated.

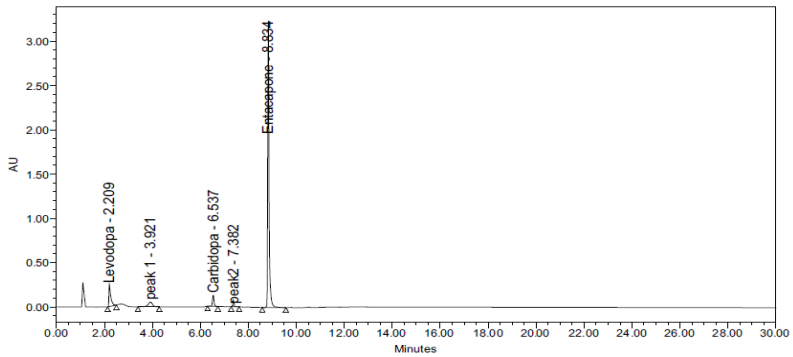
Robustness: The robustness of an analytical procedure as a measure of its capacity to remain unaffected by small, but deliberate variations in method parameters such as flow rate, column temperature and mobile phase were varied within a realistic range and the quantitative influence of the variables was determined. It provides an indication of the procedure's reliability during normal usage. It is concluded that the method is robust as it is found that the % RSD is less than 1 concerning % assay despite deliberate variations done concerning flow rate (± 0.1 ml), composition of mobile phase (±10 ml) and temperature (±5 ºC)

Stability studies: Forced degradation studies typically involved the exposure of samples of the drugs to the relevant stress conditions of acid, base, hydrolysis, oxidation, thermal, photo stability. Stability testing was established for estimating the allowed time span between sample collection and sample analysis. It is also important to evaluate an analytical method's ability to measure drug products in the presence of its degradation products.

Degradation procedure

Oxidation

To 1 ml of stock solution of Levodopa, Carbidopa and Entacapone, 1 ml of 20% hydrogen peroxide (H2O2) was added separately. The solutions were kept for 30 min at 60^0c. For HPLC study, the resultant solution was diluted to obtain 75µg/ml, 18.75 µg/ml &100µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. Figure 8.

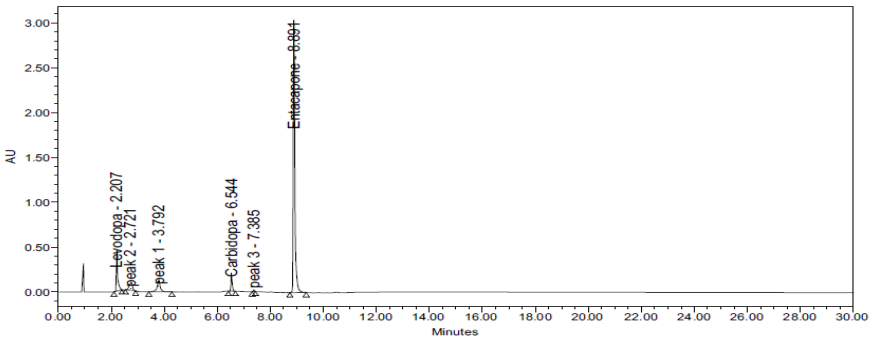


	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	Levodopa	2.209	1302725	8.90	3336	2.5	1.254	2.393
2	peak 1	3.921	701140	4.61	2324	0.9	0.588	0.777
3	Carbidopa	6.537	515183	3.69	49585	1.2	1.261	3.533
4	peak2	7.382	77894	0.51	45170	1.0	10.800	2.224
5	Entacapone	8.834	12101920	82.28	144376	1.5	8.869	44.339

Figure 8 Typical Chromatogram for Oxidation Degradation

Acid Degradation Studies

To 1 ml of stock ssolution Levodopa, Carbidopa and Entacapone, 1 ml of 2N Hydrochloric acid was added and refluxed for 30mins at 60^0c .The resultant solution was diluted to obtain 75µg/ml, 18.75 µg/ml&100µg/ml solution and 10 µl solutions were injected into the system and the chromatograms were recorded to assess the stability of sample. Figure 9.

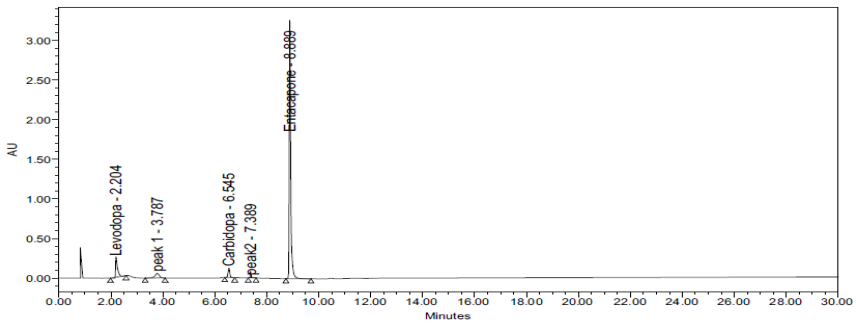


	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	Levodopa	2.207	1319979	7.91	4579	2.3	1.044	2.376
2	peak 2	2.721	242039	1.52	724	1.0	1.030	1.218
3	peak 1	3.792	769929	4.83	2538	0.9	0.489	0.694
4	Carbidopa	6.544	525570	3.55	62774	1.2	0.827	2.449
5	peak 3	7.385	43546	0.27	77635	0.9		
6	Entacapone	8.891	12156787	81.92	113723	1.6	7.264	43.627

Figure .9 Chromatogram for Acid degradation

Alkali Degradation Studies

To 1 ml of stock solution Levodopa, Carbidopa and Entacapone, 1 ml of 2N sodium hydroxide was added and refluxed for 30mins at 60^0c. The resultant solution was diluted to obtain 75µg/ml, 18.75 µg/ml &100µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. Figure 10.

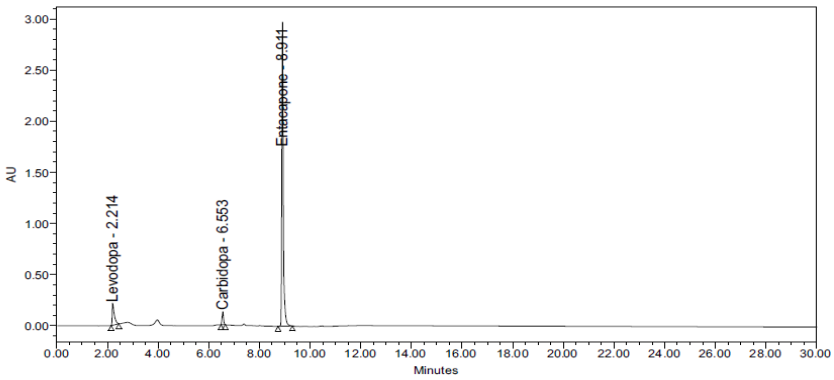


	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	Levodopa	2.204	1321295	8.90	4397	1.8	0.901	1.437
2	peak 1	3.787	690882	4.45	2580	0.9	0.525	0.754
3	Carbidopa	6.545	529598	3.54	43006	1.1	1.016	2.535
4	peak2	7.389	78282	0.50	53480	1.0	10.598	2.192
5	Entacapone	8.889	12218225	82.60	128799	1.6	8.091	42.419

Figure .10 Typical Chromatogram for Alkali Degradation

Dry Heat Degradation Studies

The standard drug solution was placed in oven at 105 °C for 6 h to study dry heat degradation. For HPLC study, the resultant solution was diluted to 75µg/ml, 18.75 µg/ml&100µg/ml solution and10µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.** Figure 11.

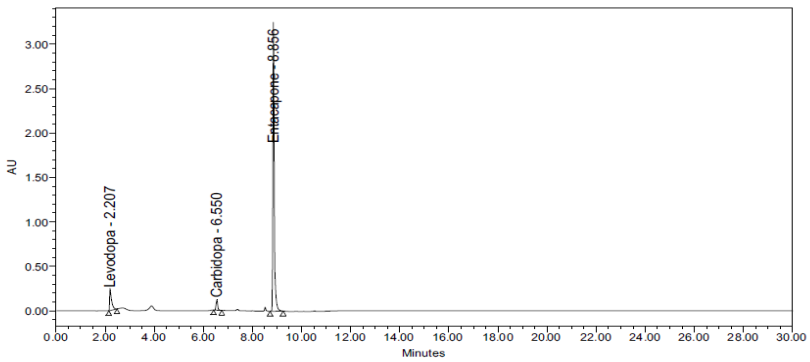


	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	Levodopa	2.214	1343817	8.75	3235	2.4	0.941	1.375
2	Carbidopa	6.553	536042	3.48	60904	1.1	0.256	0.956
3	Entacapone	8.911	12370875	87.76	111881	1.5	10.601	48.075

Figure .11 Typical Chromatogram for dry heat Degradation

Photo Stability studies

The photochemical stability of the drug was also studied by exposing the 75µg/ml, 18.75 µg/ml&100µg/ml solution to UV Light by keeping the beaker in UV Chamber for 7days or 200 Watt hours/m^2 in photo stability chamber. For HPLC study, the resultant solution was diluted to obtain 75µg/ml, 18.75 µg/ml&100µg/ml solutions and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of sample. Figure 12.

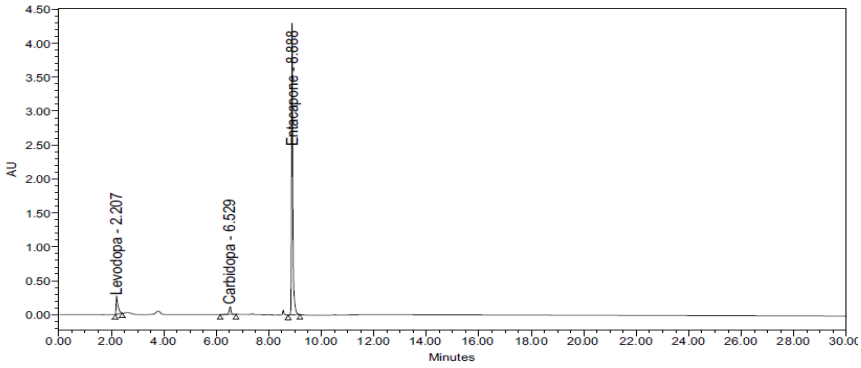


	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	Levodopa	2.207	1345356	9.38	3561	2.5	0.778	0.985
2	Carbidopa	6.550	538270	3.62	49073	1.2	0.870	0.915
3	Entacapone	8.856	12465872	87.00	138005	1.5	6.800	41.610

Figure .12 Typical Chromatogram for photo (UV Light) Degradation

Neutral Degradation Studies

Stress testing under neutral conditions was studied by refluxing the drug in water for 6hrs at a temperature of 60º. For HPLC study, the resultant solution was diluted to 75µg/ml, 18.75 µg/ml&100µg/ml solution and 10 µl were injected into the system and the chromatograms were recorded to assess the stability of the sample**.** Figure 13 and All the above Forced degradation studies values represented by Table 6.



	Peak Name	RT	Area	% Area	USP Plate Count	USP Tailing	Purity1 Angle	Purity1 Threshold
1	Levodopa	2.207	1347185	8.27	3969	2.3	1.073	2.370
2	Carbidopa	6.529	537962	3.83	39482	0.7	3.103	5.602
3	Entacapone	8.888	12426796	87.91	212114	1.4	7.169	41.291

Figure .13 Typical Chromatogram for Natural Degradation

Table 6 Forced degradation studies Results

Name of drug	Sample Treatment	% Assay	% Degradation	Purity Angle	Purity Threshold
Levodopa	**Acid**	97.65	2.35	0.44	2.376
	Base	97.74	2.26	0.901	1.437
	Peroxide	96.37	3.63	1.254	2.393
	Thermal	99.41	0.059	0.941	1.375
	Uv	99.52	0.48	0.778	0.985
	Water	99.66	0.34	1.073	2.370
Carbidopa	**Acid**	96.82	3.18	0.827	2.449
	Base	97.56	2.44	8.091	42.419
	Peroxide	94.91	5.09	1.016	2.353
	Thermal	98.75	1.25	0.256	0.956
	Uv	99.16	0.84	1.261	3.533
	Water	99.11	0.89	3.103	5.602
Entacapone	**Acid**	97.01	2.99	7.264	43.627
	Base	97.50	2.5	8.091	42.419
	Peroxide	96.57	3.43	8.869	44.339
	Thermal	98.72	1.2	10.601	48.075
	Uv	99.48	0.52	6.800	41.610
	Water	99.17	0.83	7.169	41.291

CONCLUSION

The stability indicating RP-HPLC method developed for simultaneous estimation of Levodopa, Carbidopa and Entacapone is simple, selective, rapid and precise. The simplicity and ease of operation of this duly validated method ensures that it can be successfully used for routine estimation of these three drugs simultaneously in tablet dosage formulations.

The developed and validated method is precise, accurate, isocratic and stability-indicating RP-HPLC analytical method. The method was validated for specificity, linearity, accuracy, precision, and LOD, LOQ, robustness and system suitability. No interfering peaks were found in chromatogram, indicating that the estimation of drugs is free from inference of excipients. The rapid run time of 6 min and the relatively low flow rate (1 ml/min) allows the analysis of large number of samples with less mobile phase that proves to be cost-effective. Therefore, the developed method can be used for routine analysis for simultaneous estimation and stability indicating studies of Levodopa, Carbidopa and Entacapone in bulk and pharmaceutical dosage form.

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